POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
Pol. J. Food Nutr. Sci. 2006, Vol. 15/56, No 1, pp. 49–53

INHIBITING EFFECT OF TANNIN IN CHOKEBERRY MUST ON THE WINEMAKING PROCESS

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Key words: chokeberry, wine, GPC, molecular weight

The method of high performance gel permeation chromatography (HPGPC) was used to determine molecular weights of polyphenols present in chokeberry wines. Three polyphenol fractions of the molecular weights 200, 600 and 994 Da were determined. In order to precipitate polyphenols, polyvinyl polypyrrolidone (0.2 and 0.8 g/L) and gelatin (0.2 and 0.4 g/L) were added to wines. The fermentation process of chokeberry must revealed faster completion in wines with the addition of gelatin and PVPP in contrary to wine with no additives. PVPP was shown to be more selective than gelatin in precipitating polyphenols of higher molecular weights above 600 Da, which strongly inhibit the fermentation process. In order to improve fermentation conditions, the addition of 0.8 g/L PVPP is recommended.

INTRODUCTION

Chokeberry (*Aronia melanocarpa*, Elliot), a shrub up to 2.5 m high, belongs to *Rosaceae* family originating from North America [Kalemba *et al.*, 1985]. In recent years black chokeberries have been highlighted with respect to their potential use as a food colorant and as a source of valuable phytonutrients and natural antioxidants [Benvenuti *et al.*, 2004].

Aronia melanocarpa is rich in polyphenols and particularly anthocyanins [Kalemba *et al.*, 1985]. The content of tannins amounts to 1.16% in fruits and 0.85% in pressed juice giving intensively tart, astringent flavour [Seidemann, 1993]. Tannins (polyphenols) are well known for their ability to react with proteins as they precipitate albumin, gelatin and other proteins from aqueous solutions and are used to convert animal hide into leather by cross–linking collagen molecules [Zhu *et al.*, 1997].

The formed protein–tannin complexes contribute to wine turbidity, as they fall out of a solution [Siebert *et al.*, 1996]. A factor determining the ability of tannins to bind proteins is their molecular weight. The higher the molecular weight, the higher is the affinity of tannins to proteins [Sikorski, 1996].

Polyphenols are strong inhibitors of numerous physiological enzymatic activities, due to nonspecific (and occasionally specific) protein binding ability [Yanagida *et al.*, 2003; Mitek, 1987]. These compounds inhibit many enzymes, *e.g.* acid phosphatase or α–amylase. Polyphenol compounds of high molecular weight are especially active inhibitors of pectinolytic enzymes [Gasik, 1983].

Polyphenol components are able to adsorb on the surface of yeast cells, inducing metabolic disturbances in cells. It was found that mainly the polyphenols of high molecular weight were adsorbed on cells. From 6 to 10% of polyphenols present in the must adsorb on yeast cells [Pogorzelski, 1991].

There are many methods for the separation of proanthocyanidins according to their degree of polymerization, *e.g.* thin layer chromatography (TLC) with a silica phase (qualitative method) [Lea, 1978]; column chromatographies on Sephadex G–25 [McMurrough & McDowell, 1978], BSA–Sepharose CL–4B [Oh & Hoff, 1979], Sephadex LH–20 [Boukharta *et al.*, 1988] or Fractogel TSK–HW40 [Ricardo da Silva *et al.*, 1991], Fractogel TSK 50 [Meirelles *et al.*, 1992] and frequently used HPLC (250 mm×4 mm Superspher 100 18Rp column, Merck [Sun *et al.*, 1998; Degenhardt *et al.*, 2000]; Hypersil ODS column, Agilent Technologies [Slimestad *et al.*, 2005], Zotbax SB–C18 column [Wu *et al.*, 2004]).

Gel chromatography is a separation technique in which advantage is taken of the non–ionic mechanism of a molecular sieve. Contrary to other types of chromatography, in GPC substances are separated almost exclusively according to their particle size in the solution [de Pascual–Teresa *et al.*, 1998].

In the case of condensed tannins, the GPC analysis of their acetylated derivatives is applied. Detection is carried out most frequently by UV spectrophotometry (250 to 280 nm), refractometry, or mass spectrometry analysis. The column is most often calibrated by polystyrene standards [Saucier *et al.*, 1997; Berek, 1989].

The most accurate results are obtained using acetylated derivatives because all hydroxyl groups are subject to acety-

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lation irrespective of the tannin type, and additionally, the relation between molecular weight logarithm and retention time is linear [Viriot *et al.*, 1994].

MATERIAL AND METHODS

Chemical reagents. Analytically pure acetic acid anhydride and pyridine (Sigma), analar distilled non–stabilized THF (tetrahydrofuran) eluent (POCh, Gliwice), polystyrene standards (TosoHaas, Japan), PVPP and gelatin (Begerow, P.Z.P.S. PROFOOD S.C. Sandomierz) were used. Chokeberry concentrate (64.5°Blg) was from Z.P.O.W. "Hortex" Skierniewice. Yeast *Saccharomyces cerevisiae*, Syrena species, from the Pure Culture Collection of Institute of Fermentation Technology and Microbiology of Technical University of Łódź was used in the experiment.

Preparation of wines. To activate and cultivate the yeast, a pure culture was transferred from agar slants onto sterile brewer's wort (8°Blg), and then after 48 h onto mixed chokeberry–apple must (50% of each must, 12°Blg). After 24 h the inoculation (grafting) was made on chokeberry must and after the next 24 h on the next portion of chokeberry must, which finally produced a yeast starter for 5 pitchings (0.5 g dry mass/L). All propagation stages were carried out static at a temperature 25°C.

Chokeberry must was prepared by diluting chokeberry concentrate of 64.5°Blg with water to 15°Blg. Four chokeberry pitchings were prepared. To two of them 0.2 and 0.8 g/L PVPP were added, and the next two were mixed with 0.2 and 0.4 g/L gelatin. A control sample with no PVPP and gelatin added was also prepared. The musts were prepared taking must consumption factor 0.6 L/L. To achieve wine proof of 14% vol. alcohol the original musts were sweetened with saccharose. The pitchings were inoculated with yeast of Syrena species in the amount of 5% vol. 1.5 L fermentation samples was carried out at a temperature 25°C. After the fermentation, the young wines were racked, and then filtered through Filtrox AF 70 filtration plates. After the filtration, the wines were stored for aging.

Calculations and presentation of results. The fermentation experiments were repeated three times using two parallel samples of the same kind in each. A GPC analysis of each sample was repeated twice. All chromatograms are mean values. A portion of the results was analysed statistically, and the mean value, and standard deviation were calculated.

Chemical composition of experimental wines. Basic chemical analysis (alcohol content, total extract, total acidity, volatile acidity, sugar) and sensory evaluation were carried out according to Polish Standard [PN–90/A–79120]. Polyphenols were determined with Folin–Ciocalteu method using catechin as a standard [Sejder & Datunašvili, 1972] and anthocyanins by Flueki method [Flueki & Francis, 1968].

Gel chromatography. Methods of determination were developed on the basis of the following papers: Prieur *et al.* [1994], Saucier *et al.* [1997], Viriot *et al.* [1993], and Williams *et al.* [1983].

Wine samples (50 mL each) stabilized with PVPP and gelatin and non–stabilized were frozen and then freeze–dried in a "Labconco" freeze dryer for two days. To freeze dried samples 100 mL of acetic anhrydride and pyridine mixture (1:1) was added. The reaction was carried out for one day, and then 100 mL of distilled water was added. Precipitated acetyl derivatives of tannins were filtered off and dried in a desiccator for 24 h. After drying, 5 mg of acetyl derivatives were dissolved in 1 mL THF and 20 L of the solution was injected into the chromatographic column TosoHaas TSK gel G 4000 HXL; 7.8 mm ID×300 mm; the packing diameter 6 μm. Module set from Waters consisted of pump 510; UV detector (model 486) and data processing station MAXIMA 820 was used. Detection was carried out in UV at the wavelength of λ=254 nm. Elution was performed with THF at the flow rate of 1 mL/min for *ca*. 15 min. Prior to the analysis of tannins, the system was calibrated with eleven narrow polystyrene standards (TosoHaas) of molecular weights ranging from 418 to 706 000 Da and an analytical curve of the third degree was plotted.

RESULTS AND DISCUSSION

Gel chromatographic analysis of the wines allowed us to determine molecular weights of three fractions: I – 200 Da, II – 600 Da, III – 994 Da (Table 1), which corresponds to monomers, trimers and tetramers, respectively. The degree of polymerization was determined assuming that a monomer unit of chokeberry tannins is catechin. On the chromatograms of samples with additives (Figure: 1, 2, 3, 4) as well as on standard sample (Figure 5) the peaks of these three polyphenol fractions can be seen very clearly. Chokeberry tannins are polymerized more strongly than those

TABLE 1. Molecular weight of chokeberry tannin.

Fraction	Molecular weight of acetyl derivative (Da) (mean value)	Standard deviation	Molecular weight of catechin oligomer (Da) (mean value)	Polymerization degree (mean value)
I.	423	3.03	200	1
II.	1272	6.06	600	3
III.	2105	5.79	994	4

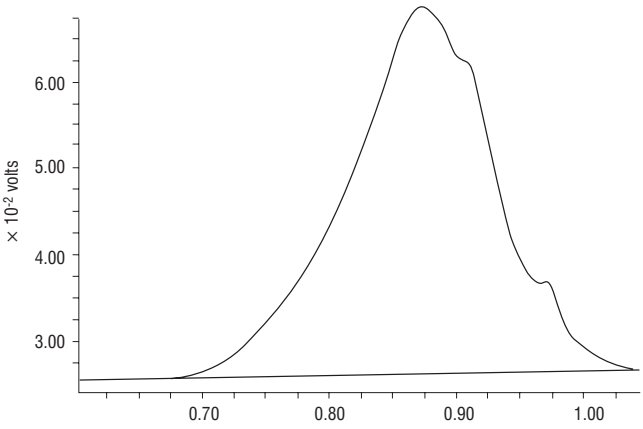

FIGURE 1. Gel chromatogram of sample 1 (wine with the addition of 0.2 g/L PVPP).

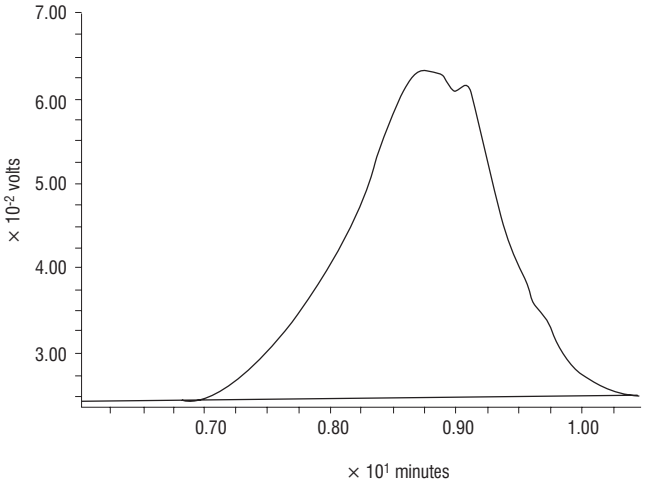

FIGURE 2. Gel chromatogram of sample 2 (wine with the addition of 0.8 g/L PVPP).

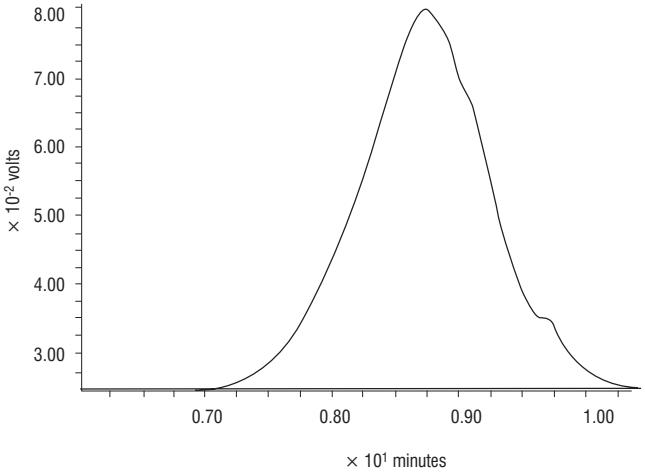

FIGURE 3. Gel chromatogram of sample 3 (wine with the addition of 0.4 g/L gelatin).

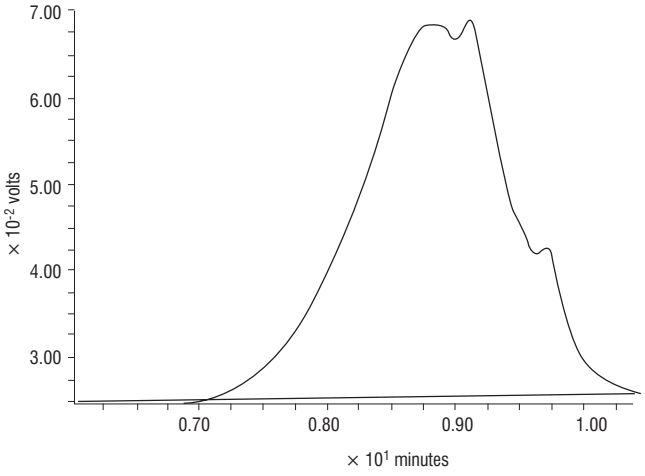

FIGURE 4. Gel chromatogram of sample 4 (wine with the addition of 0.2 g/L gelatin).

present in other fruits. Molecular weight of the largest chokeberry fraction was 994 Da, while earlier research [Pogorzelski, 1976] revealed the following molecular weights of the largest fractions: 838 Da for black currants, 540 Da for apples, and 545 Da for blueberries.

The fermentation process of chokeberry must, which

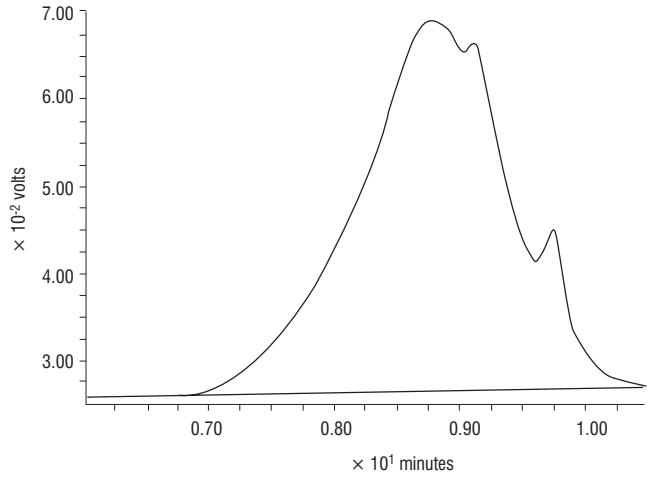

FIGURE 5. Gel chromatogram of sample 5 (wine with no additives).

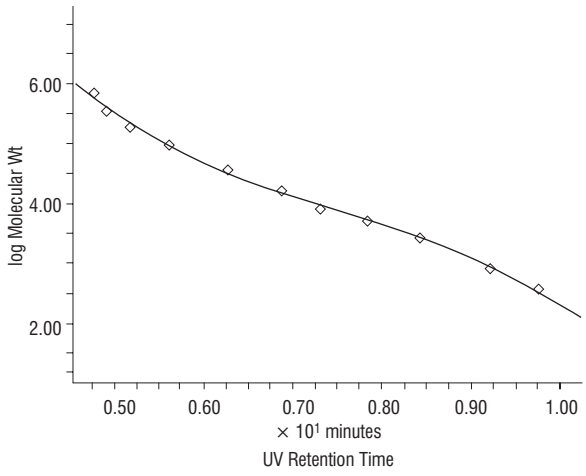

FIGURE 6. Calibration curve.

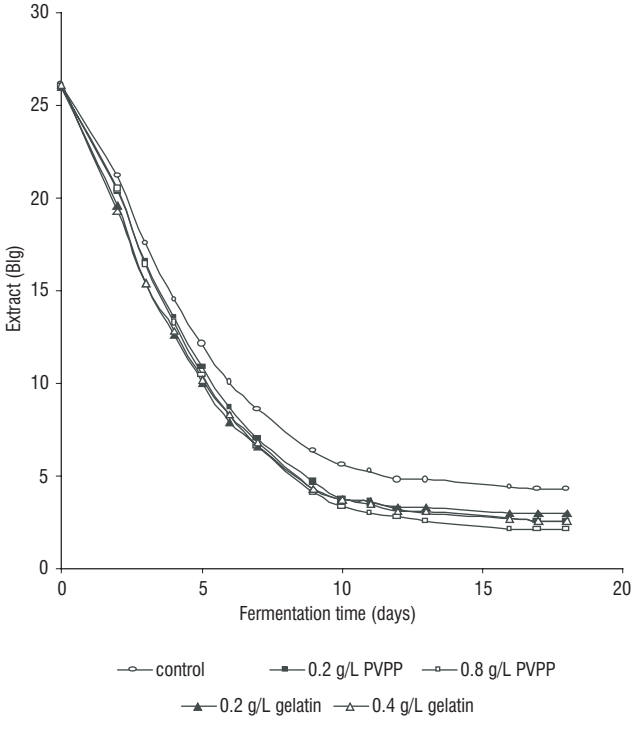

FIGURE 7. Kinetics of the fermentation process.

was monitored by a decrease in extract content, revealed faster completion in wines with the addition of gelatin and PVPP in contrary to wine with no additives (Figure 7). The received alcohol content was similar to the calculated value (14% vol.) and accounted for 13.8–14.2% vol.

The use of polyphenol adsorbents decreased polyphenol content from 4.1 g/L in wines with no additives to 3.7 g/L in wines with gelatin and PVPP (Table 2). Gelatin precipitated all polyphenol fractions from the solution, with the concentration of 0.4 g/L being much more efficient. On the chromatogram of sample 3 (Figure 3) the decrease of maximum peaks of all three polyphenol fractions can be seen more clearly than on the chromatogram of sample 4 (Figure 5). PVPP precipitated all polyphenol fractions as well, though much stronger these of higher weights (Figure 2, a significant decrease of the peak of fraction III can be observed). The fermentation process of wine with the addition of 0.8 g/L PVPP appears to proceed the most rapidly with the highest fermentation rate, which confirms the fact that polyphenol compounds of high molecular weight are especially active fermentation inhibitors.

Chokeberry must is rich in free amino acids (5.48–7.42 g/L), which stimulate the fermentation process and contribute a valuable substrate increasing flavour characteristic during maturation. Free amino acids content in chokeberry must is 10–fold higher than in apple, black currant, cherry, or in strawberry must [Czyżycki *et al.*, 1993]. On the contrary, chokeberry must also contains a large amount of tannin, which are known for their inhibiting effect on the fermentation process [Płocharski & Smolarz, 1997]. The coexistence of two opposite effects (the inhibiting effect of polyphenol and the stimulating influence of free amino acids content) in chokeberry must results in not significant differences in fermentation progress characteristic.

Noteworthy, the physicochemical analysis of wines (Table 2) reveals their conformity with Polish Standard for fruit wines, and the sensory evaluation classifies them to a group of good wines.

CONCLUSIONS

1. In this study, the inhibiting effect of polyphenols on the fermentation process were proved.

2. PVPP is a polymer binding better polyphenols than gelatin, as it precipitates more efficiently the high–molecular weight fraction of tannins above 600 Da which inhibit the process of fermentation.

3. Three polyphenol fractions of the molecular weights: 200, 600 and 994 Da were determined.

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TABLE 2. Chemical composition of experimental wines.

	Type of the probe of wine				
	Control (no additives)	PVPP (g/L)		Gelatin (g/L)	
		0.2	0.8	0.2	0.4
Sensory evaluation (point scale)	14.8(db)	16.0(db)	16.3(db)	16.3(db)	16.3(db)
Alkohol (%vol.)	13.2±0.25	14.2±0.30	14.0±0.30	13.9±0.35	13.8±0.20
Total extract (g/L)	94.13±4.34	77.09±5.13	74.94±4.92	86.6±5.67	83.45±4.05
Total acidity (g apple acid/L)	4.9±0.44	4.8±0.48	4.7±0.51	4.8±0.46	4.8±0.39
Volatile acidity (g acetic acid/L)	0.42±0.09	0.42±0.07	0.39±0.10	0.36±0.08	0.36±0.09
Reducing sugars (g glucose/L)	27.0±3.80	11.5±2.40	6.5±1.50	16.0 ±3.14	12.5±2.67
Total sugars (g glucose/L)	29.5±4.21	13.0±3.28	7.5±1.99	16.5±3.70	14.0±3.52
Saccharose (g/L)	2.5±0.99	1.0±0.58	1.0±0.49	1.0±0.46	1.0±0.41
Sugar–free extract (g/L)	64.77±7.06	64.27±6,25	67.56±6.81	70.0±7.48	69.74±5.90
Polyphenols (mg/L)	4137±31.42	4120±37.36	3705±42.22	3722±39.39	3674±45.05
Anthocyanins (mg/L)	431.0 ±11.21	438.7 ±18.75	412.9 ±21.43	418.1 ±25.79	402.6 ±14.58

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Received March 2005. Revision received September and accepted October 2005.

HAMUJĄCY WPŁYW TANIN MOSZCZU ARONIOWEGO NA PRZEBIEG FERMENTACJI WINIARSKIEJ

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W niniejszej pracy metodę chromatografii żelowej (HPGPC) wykorzystano do oznaczania mas cząsteczkowych polifenoli występujących w winach aroniowych. Oznaczono trzy frakcje polifenolowe o masach cząsteczkowych 200, 600 i 994 Da.

Zbadano wpływ tanin na przebieg procesu fermentacji winiarskiej moszczów aroniowych. W celu wytrącenia polifenoli, do win dodano żelatyny (0,2 i 0,4 g/L) oraz PVPP (0,4 i 0,8 g/L). Proces fermentacji moszczów aroniowych z dodatkiem adsorbentów polifenolowych był znacznie szybszy w porównaniu z próbą kontrolną. Wykazano, że PVPP bardziej selektywnie niż żelatyna wytrąca z win polifenole o wyższych masach cząsteczkowych – powyżej 600 Da, które w największym stopniu wpływają na hamowanie przebiegu fermentacji winiarskiej. Rekomendowane stężenie PVPP w nastawie w celu przyspieszenia przebiegu fermentacji to 0,8 g/L.